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High genetic variability of norovirus leads to diagnostic test challenges

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ABSTRACT

Background: It is important to understand the diagnostic accuracy of multiplex panels such as the Luminex xTAG® Gastrointestinal Pathogen Panel (GPP) as they are increasingly employed for routine diagnostics worldwide. Recent evaluations in our laboratory identified lower detection rates of norovirus genogroup II (NoV GII) using GPP compared to our laboratory-developed RT-qPCR, Gastroenteritis Virus Panel (GVP).
Objectives: To characterize the cases of discordant NoV GII results between GPP and GVP and determine the sensitivity of the two assays for specific NoV GII genotypes.
Study design: We genotyped discordant NoV GII strains identified in stool samples or rectal swabs collected prospectively from a cohort of children with acute gastroenteritis between December 2014 and July 2016. The sensitivities of GVP and GPP for NoV GII were compared by analyses of GVP threshold cycle (Ct) and ten-fold serial dilutions of positive samples of various NoV GII genotypes.
Results: All discordant samples (63/607) were NoV GII positive by GVP but negative by GPP. Twenty-two were successfully genotyped, fourteen of which were NoV GII genotype 2 (GII.2). The median Ct value of concordant positives was lower than that of discordant results (19.8 vs. 33.7; $P < 0.0001$). GVP was 10 and at least 10,000-fold more sensitive than GPP in detecting NoV GII.3 and GII.2, respectively, but has similar sensitivity for NoV GII.4. Discordant GII.2 variant differed genetically from concordant GII.2 variants.
Conclusions: GPP has lower sensitivity to detect NoV GII.2 than GVP and its use may lead to undetected cases clinically, and an underestimation of NoV disease burden at the population level.

1. Background

Norovirus (NoV) infection is a leading cause of gastroenteritis outbreaks and sporadic acute gastroenteritis (AGE) in individuals of all ages [1,2]. Since the implementation of rotavirus vaccine programs, NoV has been identified as the most important cause of viral AGE in young children [3–5]. To date, seven genogroups of NoV (GI–GVII) have been identified, of these, NoV GI and GII are the most important human pathogens. Norovirus GI and GII are further divided into over 40 genotypes based on the relatedness of the NoV VP1 capsid protein sequence. NoV genotyping by sequencing the RNA-dependant RNA polymerase (RdRp) is also employed to characterize isolates since recombination is an essential mechanism for NoV evolution [1,6,7]. A high degree of genetic diversity and rapid epochal evolution of NoV genotypes poses an unceasing challenge for its detection [8,9].

Reverse transcription quantitative real-time PCR (RT-qPCR) is the gold-standard approach used to detect NoV in most clinical laboratories. Recently, several commercial multiplex molecular diagnostic assays have been licensed by US Food and Drug Administration (USFDA) and Health Canada to simultaneously detect viruses, bacteria

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and parasites that cause AGE [10,11]. One of the most commonly employed platforms is the Luminex xTAG® Gastrointestinal Pathogen Panel (GPP) (Luminex Corporation, Austin Texas, US) [11]. Both the Luminex xTAG® GPP and an in-house RT-qPCR Gastroenteritis Virus Panel (GVP) were used as part of a province wide initiative designed to understand pediatric enteric infections utilizing several specimen types and testing approaches, (APPETITE – the Alberta Provincial Pediatric EnTeric Infection Team) [13]. Although high sensitivities and specificities for NoV GI and GII were reported by Luminex in their USFDA licensure application [12], as part of our APPETITE project, we detected a pattern of discordant norovirus identification between our in-house GVP and Luminex xTAG® GPP.

2. Objectives

To characterize the cases of discordant NoV GII test results between GPP and GVP and determine the sensitivity of the two assays for specific NoV GII genotypes.

3. Study design

The research proposal was approved by the ethics committees at the University of Alberta and the University of Calgary. Rectal swabs and stool samples were collected from children recruited through the APPETITE study between December 2014 and July 2016. Total nucleic acid was extracted from 300 µL of rectal swab PBS-suspension or 100–150 mg solid or 100 µL liquid stool using NucliSENS® easyMag®. Nucleic acid extracts were reverse transcribed with Invitrogen SuperScript®II and tested using GVP that simultaneously detects NoV GI and GII, rotavirus, all serotypes of adenovirus, astrovirus and sapovirus as previously described [14]; the same extracts were tested using the Luminex xTAG® GPP that detects NoV GI and GII, rotavirus, adenovirus 40/41 and 11 non-viral pathogens as per the manufacturer's instructions.

NoV test results using the two assays were compared and analyzed. A sample was classified as being discordant if NoV was detected by only one of the two study assays. All samples with discordant results were confirmed through repeat testing with both assays. A sample was deemed to have concordant results if NoV was detected by both the GPP and the GVP assays. The threshold cycle (Ct) values of the GVP assay were analyzed with a positive cut-off at 38 cycles.

The difference in the median GVP Ct values of samples with concordant and discordant results was analyzed using the Mann-Whitney *U* Test (un-paired specimens, R v3.4.0) [15]. Statistical significance was defined as two-tailed *P*-values of ≤ 0.05.

NoV GII strains from samples with confirmed discordant results were sequenced to determine the NoV GII genotypes by Sanger sequencing on region C of the VP1 gene (340 bp) followed by analyses using the NoV Genotyping tool [16]. Selected to represent each class, partial RNA-dependent RNA polymerase (RdRp) sequences (818 bp) of one NoV GII.2 concordant sample and three discordant samples were PCR amplified by using the primer pair LV4282-99F/COG-2R then LV4282-99F/G2SKR and sequenced using the Sanger method [17]. Partial RdRp sequences and sequences of region C of the VP1 gene from NoV GII.2 discordant and concordant samples were used to perform phylogenetic analysis. Sequence alignments were performed using the Muscle algorithm. Maximum likelihood trees were produced with the Kimura 2-parameter model with gamma distribution with invariant sites and 1000 bootstrap replicates using MEGA 7 [17,18]. The NoV reference sequences (M87661, U07611, AY237415, AB039775, AY502010, LC037415.1, DQ456824.1, X81879.1, KF429769, AB662902, AB067542, X86557, AB039776, AB067543, AY772730.1, KY357449, AB682733, LC145787, KJ407074.2, KX907727.1, JX445166.1, KF509946.3 and AB434770) used for comparing the similarity of sequences were obtained from GenBank (NCBI). Sequences obtained in this study were deposited in GenBank (accession numbers: MF000308–MF000321).

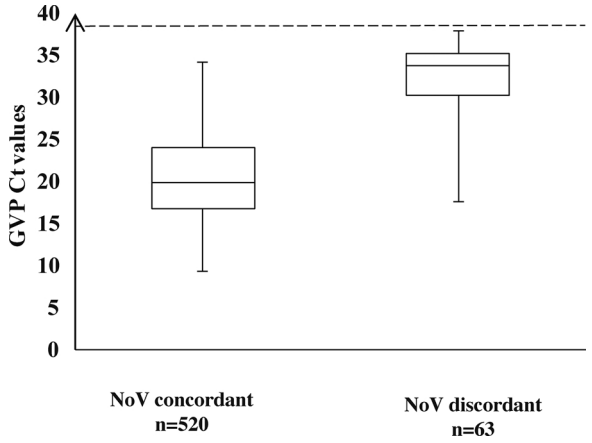

Fig 1. Boxplot comparing GVP RT-qPCR Ct value of NoV GII concordant and discordant samples. The dotted line signifies the positive cut-off at 38 cycles. Mann Whitney *U* test, $P < 0.0001$.

The sensitivity of GVP and GPP in the detection of various NoV genotypes was determined by testing ten-fold serial dilutions of four stool samples that were positive for NoV GII: a NoV GII.4_Sydney, a NoV GII.3, a NoV GII.2 from a discordant sample and a NoV GII.2 from a concordant sample.

4. Results

Of the 1470 rectal swabs and 1426 stool samples, 305 (21%) rectal swabs and 277 (19%) stool samples tested positive for NoV GII by one or both assays. The proportion of stool samples that tested positive for NoV GII by GVP and GPP were 100% (277/277) and 90% (249/277), and for rectal swab 100% (305/305) and 89% (270/305), respectively. There were 63 (11%) samples with discordant results and 520 (89%) samples with concordant results. All specimens with discordant results had tested positive for NoV GII by GVP and negative by GPP; no sample tested positive by GPP and negative by GVP. The median GVP Ct value of the samples with concordant results for Nov GII (19.8, IQR 16.7–24) was lower than those with discordant results (33.7, IQR 30–35.3) (Fig. 1, Mann-Whitney *U* Test, $P < 0.0001$).

Twenty-two (35%) of the 63 discordant samples were successfully genotyped and the remaining 41 samples (Ct ranged from 28.4 to 37.9) were not type-able due to low level of PCR amplification (Table 1). Of the 22 genotyped samples, GII.2 was identified in 14 (64%) and GII.4 Sydney in 5 (23%) samples with one each of GII.3, GII.6 and GII.17.

There was a significant difference in the GVP Ct values between NoV GII.2 (n = 14) and the NoV non-GII.2 samples (n = 8) (Mann Whitey-*U* test, $P < 0.01$) with GII.2 having a lower median Ct (27.6, range 17.6–31.7) (Table 1).

The discordant and concordant GII.2 samples were found to belong to the same GII.2 genotype using the partial VP1 sequences (Fig. 2A). One sample tested positive for NoV GII.2 by both GVP and GPP (concordant) and three discordant GII.2 samples were randomly selected for

Table 1
Genotype distributions and Ct values of NoV discordant samples.

Genotype	Number of samples	Median Ct	Min Ct	Max Ct
GII.2	14	27.6	17.6	31.7
GII.4 Sydney	5	36.2	35.5	37.2
GII.3	1	36.3		
GII.6	1	26.3		
GII.17	1	33.1		
Not genotyped	41	34.1	28.4	37.5
Total	63	–	–	–